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Receipt number	632-05-S-4466
Study number	14466

**FINAL REPORT**

Biodegradation study of microorganisms

November 11, 2005

## STATEMENT

Sponsor

Title

Biodegradation study

microorganisms

Study number

14466

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No.14466, issued on November 11, 2005).

Date

*January 19, 2006*

Study Director

## GLP STATEMENT

Sponsor

Title

Biodegradation study of

by microorganisms

Study number

14466

This study was performed in compliance with :

- (1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
- (2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

This final report reflects the raw data accurately and it has been confirmed that the test data is valid.

Date

November 11, 2005

Study Director

Signed in original

## GLP STATEMENT

Sponsor

Title Biodegradation study of xy microorganisms

Study number 14466

Amendment to the Final Report was performed in compliance with :

- (1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
- (2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

This GLP statement was issued as supplement to the statement issued on November 11, 2005 because of the amendment of the Final Report.

Date January 18, 2006

Study Director Signed in original

## QUALITY ASSURANCE STATEMENT

Sponsor

Title                      Biodegradation study                      of microorganisms

Study number              14466

It has been assured that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance as follows.

Item of inspection	Date of inspection	Date of report to Study Director and Test Facility Management
Study plan draft	September 27, 2005	September 28, 2005
Study plan	September 28, 2005	September 28, 2005
At the start of cultivation	September 29, 2005	September 29, 2005
At the middle of cultivation	October 13, 2005	October 13, 2005
At the termination of cultivation	October 27, 2005	October 28, 2005
Raw data and final report draft	November 9, 2005	November 9, 2005
Final report	November 11, 2005	November 11, 2005

Date

November 11, 2005

Quality Assurance Unit, Head

Signed in original

# QUALITY ASSURANCE STATEMENT

Sponsor

Title

Biodegradation study

microorganisms

Study number

14466

Study inspection of the corrected parts in the Final Report was carried out and it was confirmed that the correction has no problem. The result was reported to the Study Director and the Test Facility Management as follows.

Item of inspection	Date of inspection	Date of report to Study Director and Test Facility Management
Amendment to final report draft	January 17, 2006	January 17, 2006
Amendment to final report	January 18, 2006	January 18, 2006

This statement was issued as a supplement to the quality assurance statement issued on November 11, 2005.

Date

January 18, 2006

Quality Assurance Unit, Head

Signed in original

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Reference	IR spectrum supplied by sponsor

## Title

Biodegradation study of microorganisms

## Sponsor

## Test facility

## Objective

This study was performed to evaluate the biodegradability of microorganisms. by

## Test method

This study was performed according to the following test methods.

- (1) "Method for Testing the Biodegradability of Chemical Substances by Microorganisms" stipulated in the "Testing Methods for New Chemical Substances" (November 21, 2003; No. 1121002, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 13, 2003, No. 2, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121002, Environmental Policy Bureau, Ministry of the Environment)
- (2) "Ready Biodegradability: Modified MITI Test (I) (Guideline 301C, Revised July 17, 1992)" in the OECD Guidelines for Testing of Chemicals

## Applied GLP

This study complied with :

- (1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
- (2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

## Dates

Study initiation date	September 28, 2005
Experimental starting date	September 29, 2005
Experimental completion date	October 27, 2005
Study completion date	November 11, 2005

## Storage of test item, raw data, etc.

## (1) Test item

The item supplied by the sponsor is sealed in a storage vessel and stored in archives in this laboratory for ten years after the receipt of notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". If it is not stable for the storage period, it is stored as long while it is kept stable and it is disposed with approval of sponsor. Treatment of the item supplied by the sponsor after the storage period is discussed with sponsor.

## (2) Raw data and materials, etc.

Raw data, the study plan, documents concerning the study presented by the sponsor, the final report and necessary materials are stored in archives in this laboratory after the receipt of the notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of raw data and materials, etc. after the storage period is discussed with the sponsor.

## Personnel

Study Director

(1<sup>st</sup> Chemical Safety Section)Study personnel  
(Operation of biodegradation test)

Staff for cultivation of activated sludge

## Approval of final report

Study Director

Date November 11, 2005

Signature Signed in original

## SUMMARY

### Title

Biodegradation study of microorganisms

### Conditions of cultivation

- |  |          |
|--|----------|
| (1) Concentration of test item   | 100 mg/L |
| (2) Concentration of activated sludge<br>(as the concentration of suspended solid) | 30 mg/L  |
| (3) Volume of test solution  | 300 mL   |
| (4) Cultivation temperature  | 25±1 °C  |
| (5) Cultivation duration<br>(under conditions of darkness)                         | 28 days  |

### Measurement and analysis for percentage biodegradation.

- (1) Measurement of biochemical oxygen demand (BOD) with a closed system oxygen consumption measuring apparatus
- (2) Determination of dissolved organic carbon by a total organic carbon analysis (TOC)
- (3) Determination of test item by high-performance liquid chromatography (HPLC)

### Results

- |   |       |       |      |              |
|---|-------|-------|------|--------------|
| (1) Percentage biodegradation by BOD              | 6 %,  | 19 %, | 11 % | average 12 % |
| (2) Percentage biodegradation by DOC              | 11 %, | 6 %,  | 7 %  | average 8 %  |
| (3) Percentage biodegradation of test item (HPLC) | 0 %,  | 0 %,  | 0 %  | average 0 %  |

### Conclusion

The test item was not biodegraded by microorganisms under the present test conditions.

## 1. Test item

In this report, the following chemical name, etc.

1.1 Chemical name<sup>\*1</sup>1.2 Chemical structure, etc. <sup>\*1</sup>

Structural formula

Molecular formula

Molecular weight

<sup>\*1</sup> Information supplied by the sponsor

## 2. Item supplied by sponsor

### 2.1 Supplier and lot number<sup>\*1</sup>

- (1) Supplier
- (2) Lot number RS4-56

### 2.2 Purity<sup>\*1</sup>

- (1) Test item 99.5 %(w/w)
- (2) Impurity Water 0.5 %(w/w)

The test item was treated as 100 % in purity.

### 2.3 Confirmation of test item

Two infrared (IR) spectra of the test item provided by the sponsor and measured at this laboratory were confirmed to be identical (see Fig.5 and Reference).

### 2.4 Physicochemical properties<sup>\*1</sup>

- Appearance
- Melting point
- Stability
  - Stable at room temperature
  - Stable to water, dimethylsulfoxide and acetone

<sup>\*1</sup> Information supplied by the sponsor

### 2.5 Storage and stability

#### (1) Storage condition

Dark storage place at room temperature

#### (2) Stability

The test item was stable under the storage conditions, as shown by the finding that IR spectra of the test item before the experimental start and after the experimental completion were identical (see Fig.5).

### 3. Activated sludge

#### 3.1 Sludge sampling sites and date

##### (1) Sampling sites

On-site sludge sampling was carried out at the following 10 locations in Japan.

Fushikogawa city sewage plant (Sapporo-shi, Hokkaido)  
 Fukashiba industrial sewage plant (Kashima-gun, Ibaraki)  
 Nakahama city sewage plant (Osaka-shi, Osaka)  
 Ochiai city sewage plant (Shinjuku-ku, Tokyo)  
 Kitakami River (Ishinomaki-shi, Miyagi)  
 Shinano River (Niigata-shi, Niigata)  
 Yoshino River (Tokushima-shi, Tokushima)  
 Lake Biwa (Otsu-shi, Shiga)  
 Hiroshima Bay (Hiroshima-shi, Hiroshima)  
 Dokai Bay (Kitakyushu-shi, Fukuoka)

(2) Date      June, 2005

#### 3.2 Sludge sampling

##### (1) City sewage

Return sludges from sewage plants were collected.

##### (2) Rivers, lake and sea

Surface water and surface soil which was in contact with the atmosphere were collected.

#### 3.3 Preparation of activated sludge

Activated sludge was prepared as follows to maintain its uniformity.

The filtrate (5 L) of the supernatant of the activated sludge<sup>\*2</sup> cultivated about for 3 months was mixed with the mixed filtrate (5 L) of the supernatant of a sludge collected newly at each location. The mixed filtrate (10 L) was aerated<sup>\*3</sup> after the pH value of the mixture was adjusted to  $7.0 \pm 1.0$ .

\*2 The activated sludge cultivated the mixed filtrate (10 L) of the supernatant of sludge collected at the ten locations.

\*3 Prefiltered open air was used.

### 3.4 Cultivation

Roughly 30 minutes after ceasing aeration of the sludge mixture, supernatant corresponding to about 1/3 of the whole volume was removed. Dechlorinated water was added to the remaining portion so that the total volume reached 10 L. This mixture was aerated, and then a predetermined amount of synthetic sewage\*<sup>4</sup> was added to the mixture so that the concentration of the synthetic sewage was 0.1 wt% in the volume of dechlorinated water added. This procedure was repeated once every day. Cultivation was carried out at  $25 \pm 2$  °C.

#### \*4 Synthetic sewage

Glucose, peptone and potassium dihydrogenphosphate were dissolved in purified water to obtain 50 g/L of the solution for each component. The pH of the solution was adjusted to  $7.0 \pm 1.0$  with sodium hydroxide.

### 3.5 Control and use

During cultivation, the appearance of the supernatant, sedimentation of the sludge, formation of flock, pH, dissolved oxygen concentration in the solution and temperature were checked to maintain a normal state of sludge. It was confirmed that these were within the scope of the control standard stipulated in the "Testing Methods for New Chemical Substances", and these results were stored as raw data. Microflora in the activated sludge was microscopically observed and sludge with no abnormal symptoms was used for the test. The activated sludge, which was cultivated for 18.5 hours after it had been added the synthetic sewage, was used.

### 3.6 Inspection of activity and date of initiation of use of activated sludge

#### (1) Inspection of activity

Activity of the sludge was assessed using standard items.

#### (2) Date of initiation of use July 12, 2005

#### 4. Performance of biodegradation test

##### 4.1 Preparations for test

###### (1) Measurement of concentration of suspended solid

The concentration of suspended solid was measured to determine the amount of activated sludge to add.

Method	In accordance with Japanese Industrial Standards (JIS) K 0102-1998 section 14.1
Date	September 26, 2005
Result	Concentration of suspended solid in the activated sludge was 4030 mg/L.

###### (2) Preparation of basal culture medium

Each 3 mL of solutions A, B, C and D, which are prescribed in JIS K 0102-1998 section 21, were made up to 1000 mL with purified water (Takasugi Pharmaceutical Co., Ltd.), and then the pH of this solution was adjusted to 7.0.

###### (3) Reference item

Aniline (reagent grade, Showa Chemicals Co., Ltd. Lot No. SP-3442Z) was used as a reference item to confirm that the sludge was sufficiently active.

#### 4.2 Preparation of test solutions

The following test solutions were prepared and cultivated under the conditions described in section 4.3.

##### (1) Addition of test item or aniline

###### (a) Test solution (water + test item) (n=1, Vessel No.1)

In one test vessel, 297 mL of purified water and 3 mL of 10.0 g/L of the test item in water was added, so that the concentration of the test item reached 100 mg/L and then the pH of the solution was measured. 10.0 g/L of the test item in water was pretreated as follows. The item supplied by the sponsor was accurately weighed and dissolved in purified water to obtain it.

###### (b) Test solution (sludge + test item) (n=3, Vessel No.2, 3 and 4)

In each test vessel, the basal culture medium (the volume was less than 297 mL by the volume (2.23 mL) of activated sludge inoculated) and 3 mL of 10.0 g/L of the test item in water was added, so that the concentration of the test item reached 100 mg/L. The pH of the solution was measured. 10.0 g/L of the test item in water was pretreated as follows. The item supplied by the sponsor was accurately weighed and dissolved in purified water to obtain it.

###### (c) Test solution (sludge + aniline) (n=1, Vessel No.6)

In one test vessel, the basal culture medium (the volume was less than 300 mL by the volume (2.23 mL) of activated sludge inoculated) and aniline were added, so that the concentration reached 100 mg/L.  $29.5 \mu\text{L}$  [ $30 \text{ mg} = 29.5 \mu\text{L} \times 1.022 \text{ g/cm}^3$  (density)] of aniline was taken out with microsyringe and added.

###### (d) Test solution (control blank) (n=1, Vessel No.5)

In one test vessel, nothing was added to the basal culture medium (the volume was less than 300 mL by the volume (2.23 mL) of activated sludge inoculated).

##### (2) Inoculation of activated sludge

The activated sludge cultivated under the conditions described in section 3 was added to each test vessel, (b), (c) and (d), so that the concentration of the suspended solid reached 30 mg/L.

#### 4.3 Instruments and conditions of cultivation

##### (1) Instruments for cultivation

Closed system oxygen consumption measuring apparatus  
(Temperature controlled bath and measuring unit :

Asahi Technicon Co., Ltd.)

(Data sampler : Asahi Technicon Co., Ltd.)

Vessel 300 mL in volume (improved type)

Absorbent for carbon dioxide

Soda lime No.1 (for absorption of carbon dioxide,  
Wako Pure Chemical Industries, Ltd.)

##### (2) Conditions of cultivation

Cultivation temperature  $25 \pm 1$  °C

Cultivation duration 28 days (under conditions of darkness)

Stirring method Each test solution was stirred by a magnetic stirrer.

##### (3) Room

Apparatus room A

#### 4.4 Observation and measurement of test conditions

##### (1) Observation of test solution

During the cultivation, the appearance of the test solution was observed periodically and conditions of the instruments were checked properly.

##### (2) Measurement of biochemical oxygen demand (BOD)

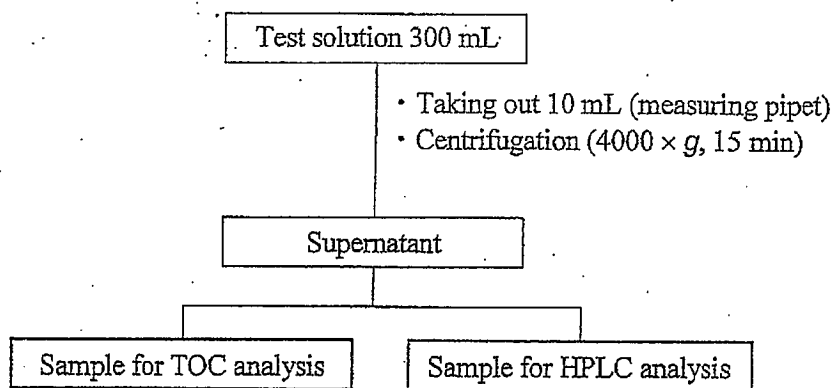
During the cultivation period, the change in BOD of the test solutions was measured by autorecording using a data sampler. Cultivation temperature was measured and recorded once a day.

#### 4.5 Analysis of test solution

After the termination of the cultivation, dissolved organic carbon and the test item in the test solutions were determined. The pH of the test solution (water + test item) and the test solutions (sludge + test item) was measured.

##### 4.5.1 Pretreatment of test solutions for analysis

After the termination of the cultivation, the test solution (water + test item), the test solutions (sludge + test item) and the test solution (control blank) were pretreated for total organic carbon (TOC) analysis on dissolved organic carbon and high-performance liquid chromatography (HPLC) analysis of the test item as follows.



#### 4.5.2 Quantitative analysis

##### (1) Determination of dissolved organic carbon

The dissolved organic carbon (DOC) in the samples for TOC analysis was analyzed under the following conditions.

The concentration of DOC was calculated by subtracting concentration of the inorganic carbon (IC) from concentration of the total carbon (TC). The concentration of TC and IC in the test solutions was calculated proportionally from the peak area of the test solution by comparison with that of 80.0 mgC/L standard solution for TC analysis and 80.0 mgC/L standard solution for IC analysis, respectively (see Table-2). The standard solution for TC analysis was prepared by dissolving potassium hydrogenphthalate in purified water. The standard solution for IC analysis was prepared by dissolving sodium hydrogencarbonate and sodium carbonate in purified water.

The concentration of dissolved organic carbon corresponding to the minimum determination limit was regarded as 1.0 mgC/L.

##### Analytical conditions

Instrument	Total organic carbon analyzer Shimadzu Corporation type TOC-5000A
Temperature of furnace	680 °C
Flow rate	150 mL/min
Injection volume	33 µL
Sensitivity	Range 5

## (2) Determination of test item

The samples for HPLC analysis were analyzed under the following conditions. The concentration of the test item in the sample for HPLC analysis was calculated proportionally by comparing the peak area on the chromatogram of the sample for HPLC analysis with that on the chromatogram of 100 mg/L standard solution (see Table-3 and Fig.3).

The lowest detectable peak area of the test item was regarded as 580  $\mu\text{V}\cdot\text{sec}$  considering the noise level, which corresponded to the test item concentration of 0.99 mg/L.

## (a) Analytical conditions

Instrument	High-performance liquid chromatograph Shimadzu Corporation type LC-2010A
Column	L-column ODS (15 cm $\times$ 2.1 mm I.D., Chemicals Evaluation and Research Institute, Japan)
Column temperature	40 $^{\circ}\text{C}$
Eluent	A (55 %) : Acetonitrile B (45 %) : Water <sup>*5</sup> / 0.5 mol/L tetra- <i>n</i> - butylammonium phosphate (100/2 V/V)
Flow rate	0.2 mL/min
Measurement wavelength	
Sample size	8 $\mu\text{L}$
Detector output	2 V/AU

\*5 City water was treated by Ultra pure water system.

## (b) Preparation of standard solution

The standard solution to determine the concentration of the test item in the sample for HPLC was prepared as follows.

100 mg of the item supplied by the sponsor was accurately weighed and dissolved in purified water to obtain 1000 mg/L solution of the test item. 100 mg/L standard solution was then prepared from this solution by dilution with purified water.

## (c) Calibration curve

25.0, 50.0 and 100 mg/L standard solutions were prepared by the same method as described in (b). These solutions were analyzed according to the analytical conditions described in (a). A calibration curve was drawn based on the relation between the peak area on the chromatograms and the respective concentrations (see Fig.2).

#### 4.6 Calculation of percentage biodegradation

The percentage biodegradation was calculated by the following equations and rounded off to the whole number.

##### (1) Percentage biodegradation by BOD

$$\text{Percentage biodegradation (\%)} = \frac{\text{BOD} - \text{B}}{\text{TOD}^{*6}} \times 100$$

BOD : Biochemical oxygen demand in the test solution  
(sludge + test item) (experimental) (mg)

B : Biochemical oxygen demand in the control blank  
(experimental) (mg)

TOD<sup>\*6</sup> : Theoretical oxygen demand required when the test  
item was completely oxidized (theoretical) (mg)

\*6 The purity was regarded as 100 % and TOD was calculated.

##### (2) Percentage biodegradation by DOC

$$\text{Percentage biodegradation (\%)} = \frac{\text{DOCw} - \text{DOCs}}{\text{DOCw}} \times 100$$

DOCs : Residual amount of the dissolved organic carbon in the test  
solution (sludge + test item) (experimental) (mgC)

DOCw : Residual amount of the dissolved organic carbon in the test  
solution (water + test item) (experimental) (mgC)

##### (3) Percentage biodegradation of test item

$$\text{Percentage biodegradation (\%)} = \frac{\text{Sw} - \text{Ss}}{\text{Sw}} \times 100$$

Ss : Residual amount of the test item in the test solution  
(sludge + test item) (experimental) (mg)

Sw : Residual amount of the test item in the test solution  
(water + test item) (experimental) (mg)

#### 4.7 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401:1999 rule B.

### 5. Validity of test conditions

The validity criteria of the test and the values in the present test are shown in the following table. The present test was valid because all of the values in the present test met the criteria.

		Value in present test	Value of criterion	See
Difference of extremes of values of percentage biodegradation	Percentage biodegradation by BOD	13 %	< 20 %	7.3 Percentage Biodegradation
	Percentage biodegradation by DOC	5 %		
	Percentage biodegradation of test item	0 %		
Percentage biodegradation of aniline by BOD	After 7 days	74 %	$\geq 40$ %	Table-1 Fig.1
	After 14 days	76 %	$\geq 65$ %	
BOD value of control blank	After 28 days	8.3 mg	< 18 mg ( < 60 mg/L)	Table-1 Fig.1

### 6. Factors possibly affecting accuracy

No adverse effects on the reliability of this test were noted.

## 7. Results

### 7.1 Appearances of test solutions

Appearances of test media in cultivation vessels were as follows.

	Test solution	Appearance	pH
At the start of cultivation	Water + test item	The test item was dissolved. The test solution was colorless.	Vessel-1 5.9
	Sludge + test item	The test item was dissolved. The test solutions were colorless.	Vessel-2 7.0 Vessel-3 7.0 Vessel-4 7.0
At the termination of cultivation	Water + test item	Insoluble compound was not observed. The test solution was colorless.	Vessel-1 6.6
	Sludge + test item	Insoluble compound except the sludge was not observed. The test solutions were colorless. Growth of the sludge was not observed.	Vessel-2 7.3 Vessel-3 7.3 Vessel-4 7.3

### 7.2 Analytic results of test solutions

Analytic results of the test solution after 28 days were as follows.

In the test solution (water + test item) and the test solutions (sludge + test item), theoretical amount of the test item remained and no peak except the test item was detected on the HPLC chromatogram. Then, it was judged that any converted products were not produced. Therefore, converted products were not analyzed.

		Water + test item	Sludge + test item				Theoretical amount	Table	Fig.
		Vessel-1	Vessel-2	Vessel-3	Vessel-4				
BOD <sup>*7</sup>	mg	0.7	0.9	3.1	1.8	15.9		1	1
Residual amount and percentage residue <sup>*7</sup> of DOC	mgC	6.3	5.7	5.9	5.9	6.0		2	-
	%	106	95	99	98	-			
Residual amount and percentage residue of test item (HPLC)	mg	30.0	30.0	30.0	30.0	30.0		3	3
	%	100	100	100	100	-			

<sup>\*7</sup> The value of control blank was subtracted from the values of the test solutions (sludge + test item).

### 7.3 Percentage biodegradation

Percentage biodegradations after 28 days were as follows.

		Sludge + test item				Table
		Vessel-1	Vessel-2	Vessel-3	Average	
Percentage biodegradation by BOD	%	6	19	11	12	1
Percentage biodegradation by DOC	%	11	6	7	8	2
Percentage biodegradation of test item (HPLC)	%	0	0	0	0	3

### 7.4 Discussion

It is considered that the test item was not biodegraded because theoretical amount of the test item was detected by the HPLC analysis. However, the percentage biodegradations by BOD were calculated as 6 %, 19 % and 11 %. The reason is considered that the TOD of the test item is small (15.9 mg) and the BOD value of the control blank was about 50 % of the TOD (8.3 mg), and then small difference of the BOD values between the test solutions strongly influenced to the percentage of the biodegradation by BOD. The percentage biodegradations by DOC were calculated as 11 %, 6 % and 7 %. The reason is considered that small difference of the analytical values was calculated as large values of the percentage biodegradation by DOC because theoretical amount of DOC is small (6.0 mgC).

### 7.5 Conclusion

The test item was not biodegraded by microorganisms under the present test conditions

## 8. Remarks

## 8.1 Instruments used for test

Fourier transform infrared spectrophotometer :  
Shimadzu Corporation type IRPrestige-21  
Closed system oxygen consumption measuring apparatus :  
see page 11  
Total organic carbon analyzer : see page 13  
High-performance liquid chromatograph :  
see page 14  
Electronic analytical balance : Sartorius AG type BP210S  
pH meter : Toa Electronics Ltd. type HM-50G  
Ultraviolet and visible spectrophotometer :  
JASCO Corporation type V-560  
Refrigerated centrifuge : KUBOTA Manufacturing Corporation  
type 5922

## 8.2 Reagents used for analysis

Acetonitrile (HPLC grade) : Wako Pure Chemical Industries, Ltd.  
Purified water : Takasugi Pharmaceutical Co., Ltd.  
0.5 mol/L Tetra-*n*-butylammonium phosphate (ion-pair reagent for HPLC) :  
Tokyo Kasei Kogyo Co., Ltd.  
Potassium hydrogen phthalate (reagent grade) :  
Wako Pure Chemical Industries, Ltd.  
Sodium hydrogencarbonate (reagent grade):  
Wako Pure Chemical Industries, Ltd.  
Sodium carbonate (reagent grade): Wako Pure Chemical Industries, Ltd.

Table-1 Calculation table for percentage biodegradation by BOD

Study No. 14466		Duration of cultivation: 28 days							
Vessel No.	7th day		14th day		21st day		28th day		Mean Deg. (%)
	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	
[6]	70.5	74	75.9	76	77.4	77	77.4	77	
[5]	3.5	-	7.0	-	8.3	-	8.3	-	
[2]	4.3	5	8.2	8	9.2	6	9.2	6	12
[3]	5.3	11	10.3	21	11.4	19	11.4	19	
[4]	4.7	8	9.1	13	10.1	11	10.1	11	
[1]	0.0	-	0.0	-	0.7	-	0.7	-	

Deg. : Percentage biodegradation

Vessel No. [6] : Sludge + aniline  
Vessel No. [5] : Control blank [B]  
Vessel No. [2] [3] [4] : Sludge + test item  
Vessel No. [1] : Water + test item

Test item of 30.0 mg was added.

Chart of BOD : Fig. 1

Deg. =  $[\text{BOD} - \text{B}] / [\text{TOD}] \times 100 (\%)$

TOD of test item :

TOD of aniline : 90.3 (mg)

$\text{C}_6\text{H}_7\text{N} + 8.75 \text{ O}_2 \rightarrow 6 \text{ CO}_2 + 3.5 \text{ H}_2\text{O} + \text{NO}_2$

$8.75 \text{ O}_2 / \text{C}_6\text{H}_7\text{N} = 279.99 / 93.13 = 3.01$

$\text{TOD} = 30 \times 3.01 = 90.3 (\text{mg})$

Oct.27,2005 Name

Table-2 Calculation table for percentage biodegradation by DOC

Study No. 14466

Sample description	A	B	C	D	E
Water blank	n.d.				
[1] Water + test item	21.16	6.3	106		
[2] Sludge + test item	20.83	5.7	95	11	
[3] Sludge + test item	21.71	5.9	99	6	8
[4] Sludge + test item	21.51	5.9	98	7	
[5] Control blank	1.91				
<p>Amount of test item added : 30.0 (mg)</p> <p>Volume of test solution : 300 (mL)</p> <p>Theoretical amount of carbon : 6.0 (mgC) <math>(30.0) \times (6C / C_6H_4F_{11}NO_4)</math></p> <p>A : Measured value (mgC/L)</p> <p>B : Amount of DOC</p> <p><math>B_w = (A(\text{Water} + \text{test item}) - A(\text{Water blank})) \times 300 / 1000 \text{ (mgC)}</math></p> <p><math>B_s = (A(\text{Sludge} + \text{test item}) - A(\text{Control blank})) \times 300 / 1000 \text{ (mgC)}</math></p> <p>C : Percentage residue (%)</p> <p><math>C = B / (\text{Theoretical amount of carbon}) \times 100</math></p> <p>D : Percentage biodegradation (%)</p> <p><math>D = (B_w - B_s) / B_w \times 100</math></p> <p>E : Average percentage biodegradation (%)</p>					

October 28, 2005 Name

Table-3 Calculation table for percentage biodegradation of test item

Study No. 14466

Sample description	A	D	E	F	G
Standard solution 100mg/L	58347				
[1] Water + test item	58382	30.0	100		
[2] Sludge + test item	58384	30.0	100	0	
[3] Sludge + test item	58368	30.0	100	0	0
[4] Sludge + test item	58397	30.0	100	0	
[5] Control blank	n.d.				

Amount of test item added : 30.0 (mg)

A : Peak area ( $\mu\text{V}\cdot\text{sec}$ )

B : Final volume : 10 (mL)

C : Ratio of portion used for analysis : 10/300

D : Residual amount of test item (mg)

$$D_w = H \times (A(\text{Water} + \text{test item}) / A(\text{Standard})) \times (B / C) / 1000$$

$$D_s = H \times \{ (A(\text{Sludge} + \text{test item}) - A(\text{Control blank})) / A(\text{Standard}) \} \times (B / C) / 1000$$

E : Percentage residue (%)

$$E = D / 30.0 (\text{mg}) \times 100$$

F : Percentage biodegradation (%)

$$F = \{ (D(\text{Water} + \text{test item}) - D(\text{Sludge} + \text{test item})) / D(\text{Water} + \text{test item}) \} \times 100$$

G : Average percentage biodegradation (%)

H : Concentration of standard solution : 100 (mg/L)

See Fig. 3

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Name

Noriko Miyaura

Study No. 14466 ( Test item \_\_\_\_\_ )

Cultivating conditions:

Concentration

Test item ..... 100 (mg/L)

Reference item (aniline) ..... 100 (mg/L)

Activated sludge ..... 30 (mg/L)

Temperature .....  $25 \pm 1$  °C

Duration ..... 28 days (Sep.29,2005 - Oct.27,2005)

Note: —

Vessel No.	Sample Description	BOD (mg)			
		7th day	14th day	21st day	28th day
[1]	Water + test item	0.0	0.0	0.7	0.7
[2]	Sludge + test item	4.3	8.2	9.2	9.2
[3]	Sludge + test item	5.3	10.3	11.4	11.4
[4]	Sludge + test item	4.7	9.1	10.1	10.1
[5]	Control blank [B]	3.5	7.0	8.3	8.3
[6]	Sludge + aniline	70.5	75.9	77.4	77.4

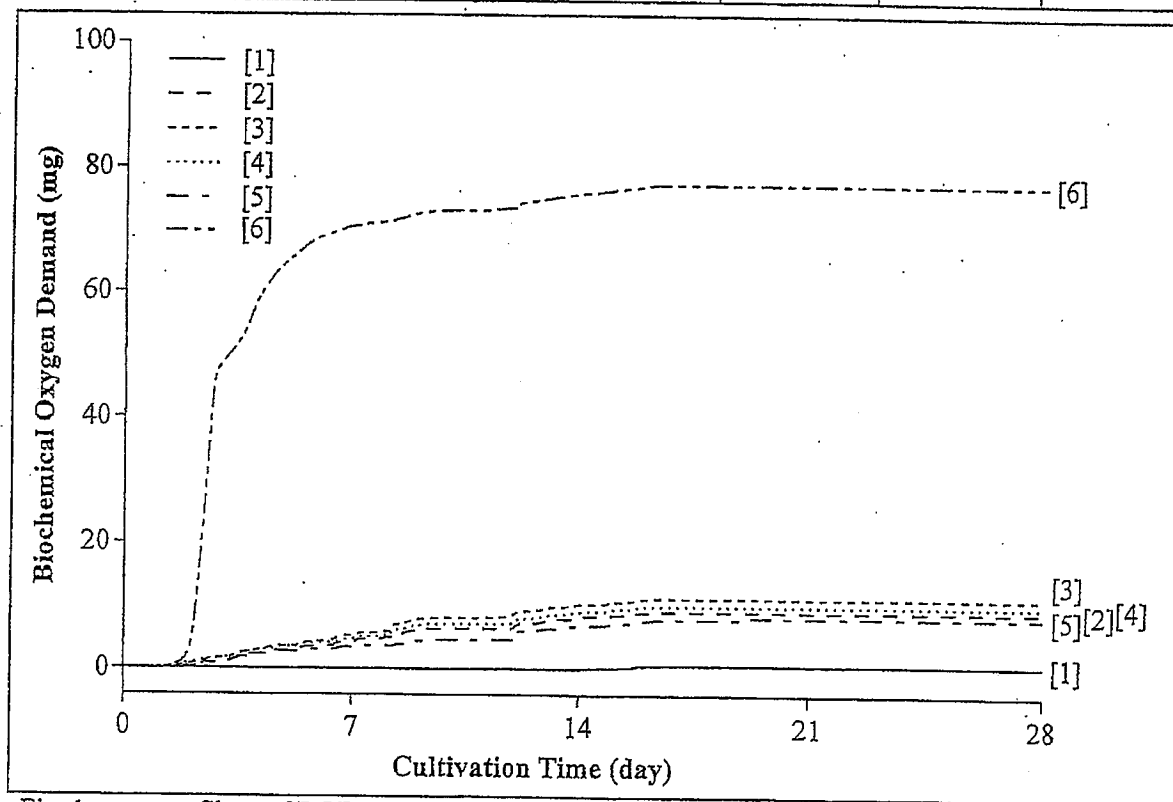


Fig. 1 Chart of BOD.

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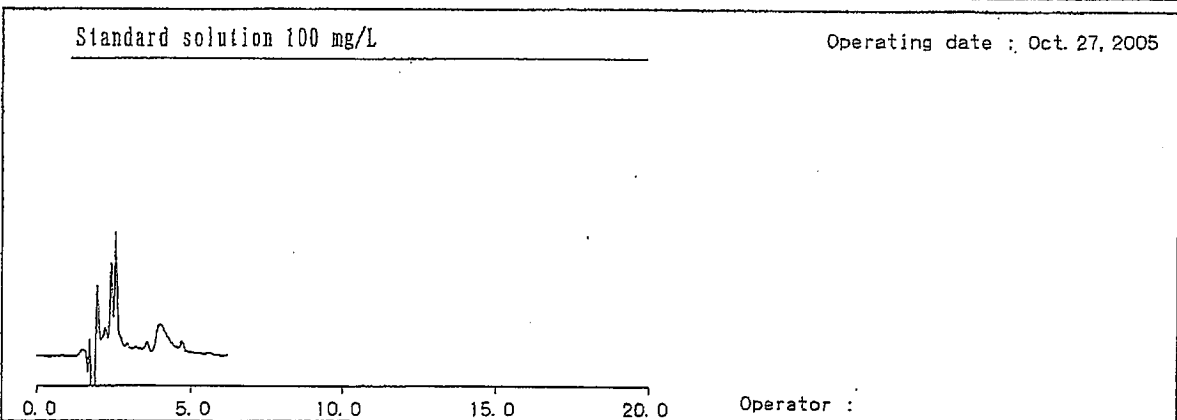
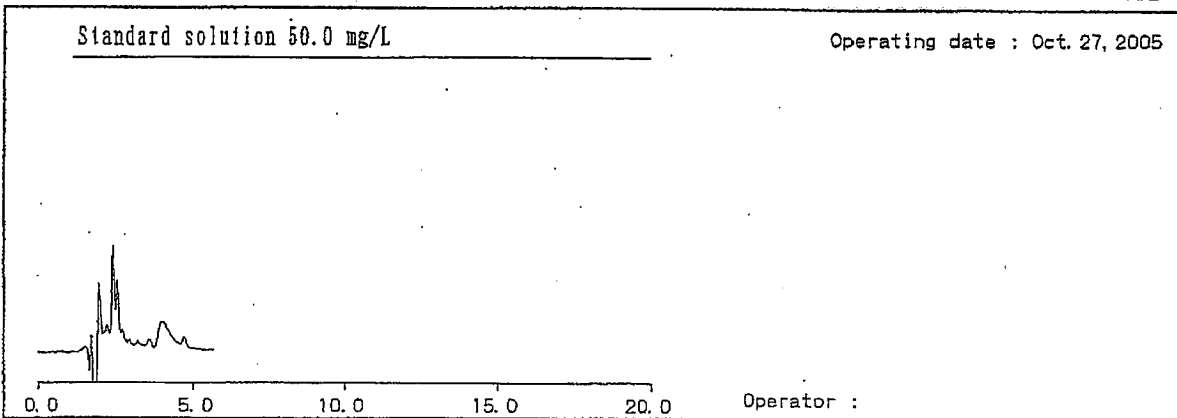
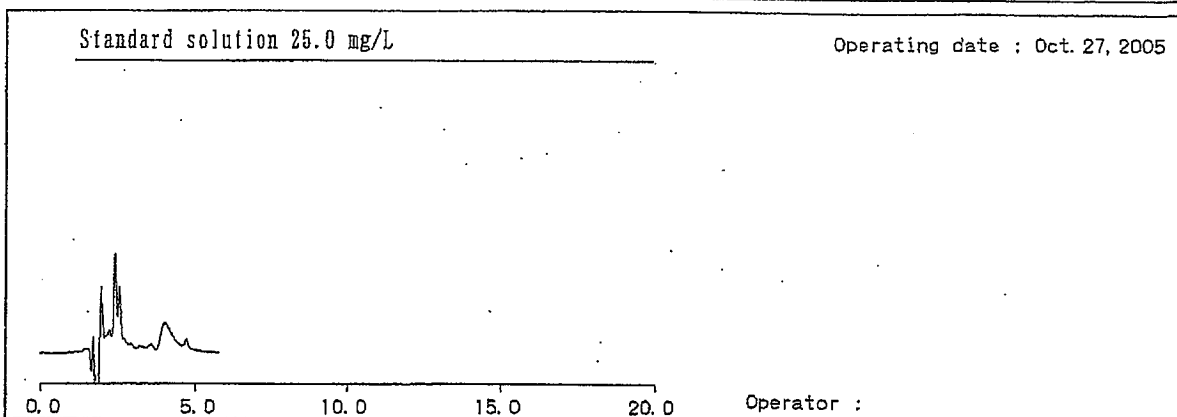
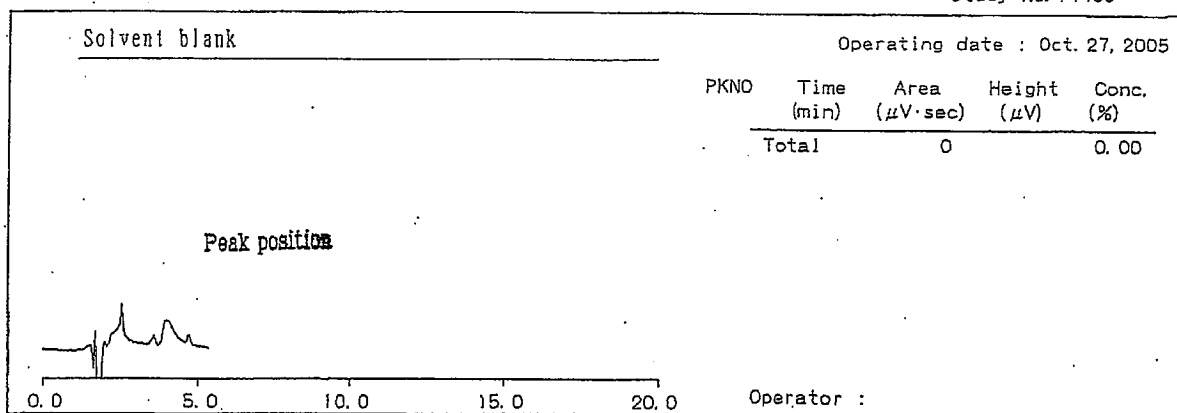
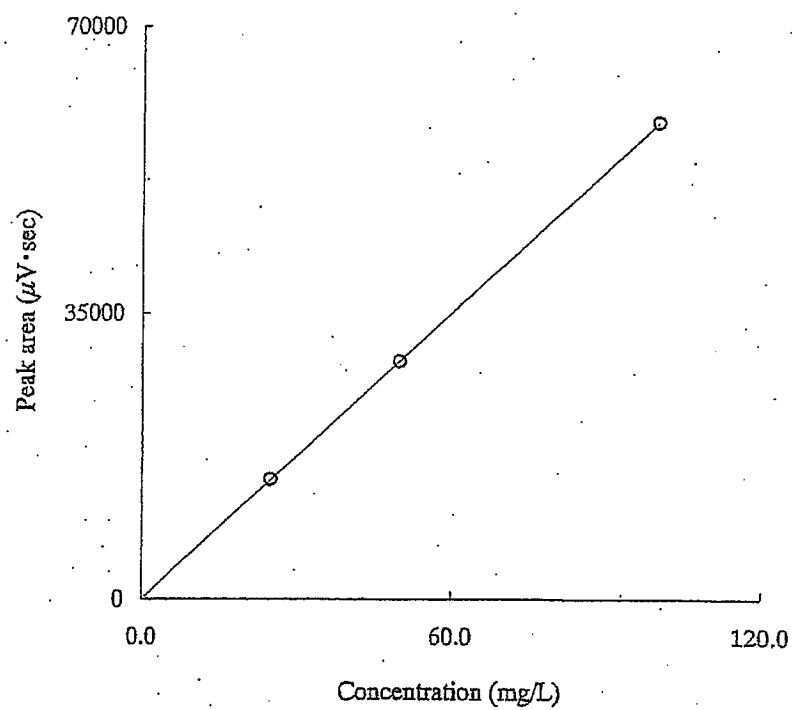


Fig. 2 - 1 Chromatograms of HPLC analysis for calibration curve.

Date : Oct. 27, 2005

Name : Noriko Miyaura



$$y = 583x$$

$$r = 1.00$$

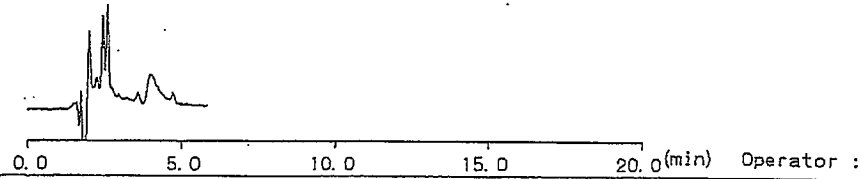
Fig. 2 - 2 Calibration curve of test item.

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Name

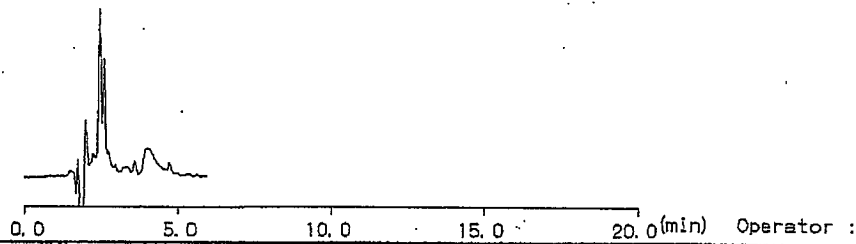
Standard solution 100 mg/L

Operating date : Oct. 27, 2005



[ 1 ] Water + test item

Operating date : Oct. 27, 2005



[ 2 ] Sludge + test item

Operating date : Oct. 27, 2005

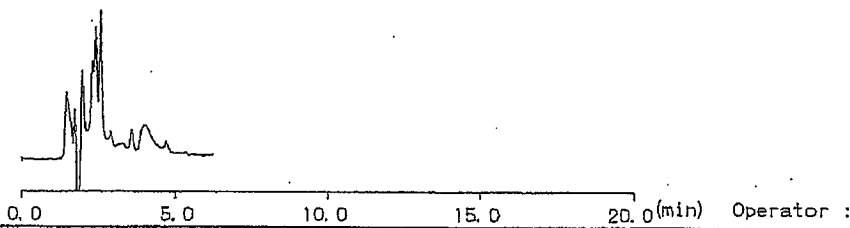


Fig. 3 - 1 Chromatograms of HPLC analysis for test solution.

Date : Oct. 27, 2005

Name :

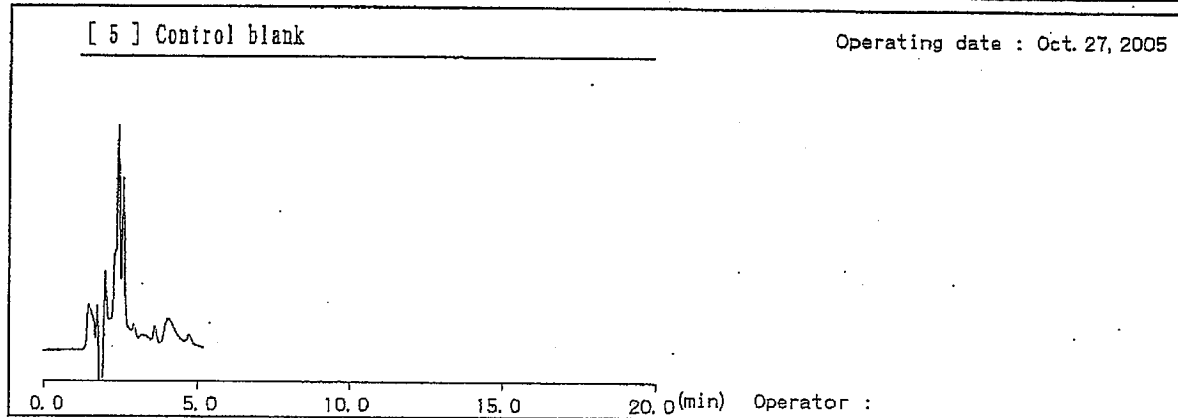
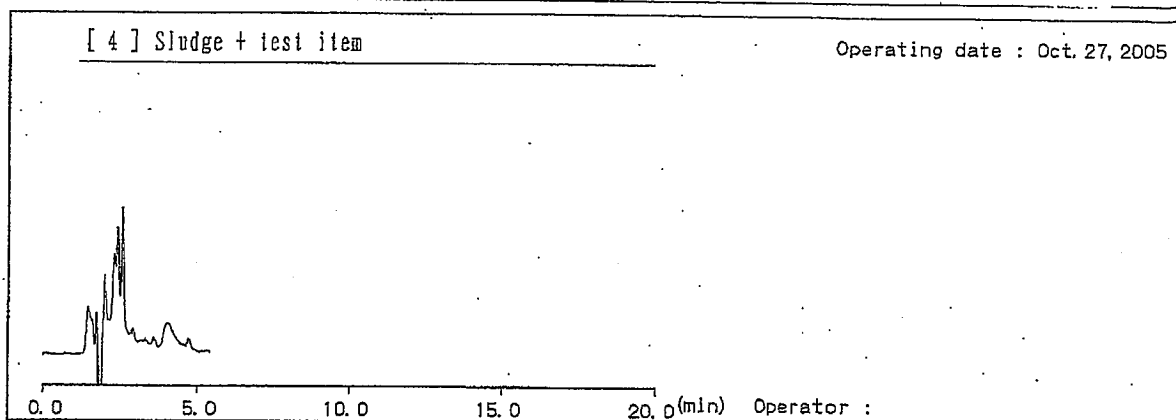
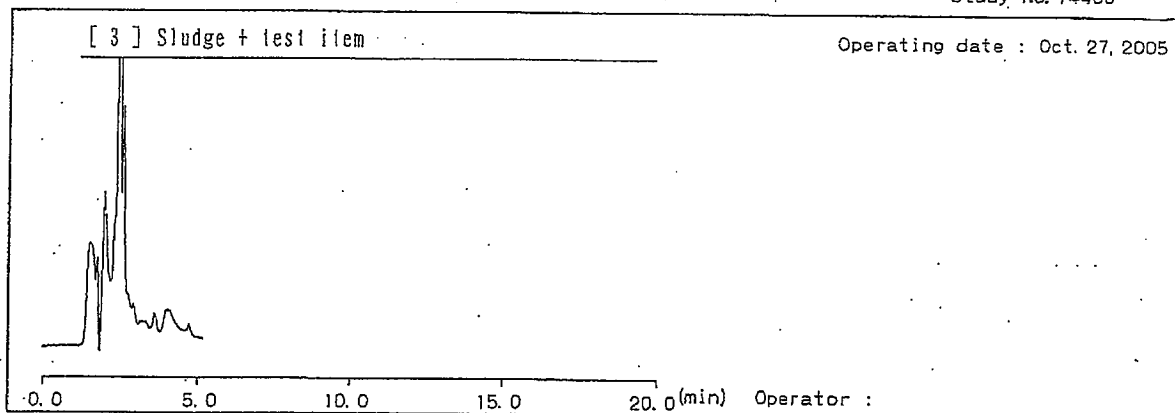


Fig. 3 - 2 Chromatograms of HPLC analysis for test solution.

Date : Oct. 27, 2005

Name :

Study No.	14466
Date	Aug. 4, 2005
Sample	Test item
Solvent	Purified water
Reference	-
Cell	10mm x 10mm, quartz
Instrument	JASCO V-560
Photometric Mode	Abs
7/17=2/17E 2005. 8. 4 5 5 5 5	

Chemicals Evaluation and Research Institute, Japan Kurume Laboratory

Wavelength[nm]

Fig. 4 UV spectrum of test item.

Instrument : Shimadzu IRPrestige-21  
Study No. : 14466  
Sample : Test item  
Method : ATR  
Date : September 26, 2005  
Name :

Fig. 5 - 1 IR spectrum of test item measured before experimental start.

Instrument : Shimadzu IRPrestige-21  
Study No. : 14466  
Sample : Test item  
Method : ATR  
Date : October 28, 2005  
Name

Fig. 5 - 2 IR spectrum of test item measured after experimental completion.

Reference

IR spectrum supplied by sponsor.

## Amendment to Final Report

1. Title                      Biodegradation study                      microorganisms
2. Study number            14466
3. Content                   Sponsor                      (page 1)
4. Reason                    Because there was an error to a zip code of the sponsor, and there were instructions of a correction from the sponsor.
5. Content of correction    " 262-0032 " is corrected to " 290-8566 ".

### 6. Approval

Date                      January 18, 2006

Study Director                      Signed in original